Electrochemistry-Mass Spectrometry in Drug Metabolism and Protein Research

Hjalmar P. Permentier[1*], Andries P. Bruins[1] and Rainer Bischoff[2]

[1]*Mass Spectrometry Core Facility, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands;* [2]*Analytical Biochemistry, University Centre for Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

Abstract: The combination of electrochemistry coupled on-line to mass spectrometry (EC-MS) forms a powerful analytical technique with unique applications in the fields of drug metabolism and proteomics. In this review the latest developments are surveyed from both instrumental and application perspectives. The limitations and solutions for coupling an electrochemical system to a mass spectrometer are discussed. The electrochemical mimicking of drug metabolism, specifically by Cytochrome P450, is high-lighted as an application with high biomedical relevance. The EC-MS analysis of proteins also has promising new applications for both proteomics research and biomarker discovery. EC-MS has furthermore advantages for improved analyte detection with mass spectrometry, both for small molecules and large biomolecules. Finally, potential future directions of development of the technique are briefly discussed.

Key Words: Electrochemistry, mass spectrometry, drug metabolism, protein oxidation.

1. INTRODUCTION

Electrochemistry (EC) is a very broad and mature field with many applications in the biological and biomedical fields. The most familiar biological application is detection for liquid separation methods and in biosensors. The on-line coupling of electrochemical flow-through cells allows combination with other detection techniques, but interfacing with a mass spectrometer (EC-MS) is a relatively new development. Although the first advances were made some 20 years ago, with work by Hambitzer [1] and Brajter-Toth and coworkers [2], this combination of analytical methods has found rather limited application up to date. This could be due to the initial practical problems in interfacing EC and MS, the unfamiliarity of researchers working in either field with the other field, and the lack of well-defined, published applications. With more suitable instrumentation, and by solving a number of technical problems, there has been a significant increase in interest in recent years, catalyzed by the need for new solutions for metabolomics and proteomics [3]. In this review we will survey the recent work on instrumental and method development (updating the review of Diehl *et al*. [4]), and some practical applications of biomedical interest. We will also give an overview of related areas of research where the combination of the two techniques has been proven, or has the clear potential, to be useful.

The choice of using a mass spectrometer as an analytical detector in combination (usually in series) with an electrochemical detector or as the detector of electrochemical reaction products, is a natural one, since it provides complementary information. Despite recent technical advances, mass spectrometers still cannot reach the sensitivity of electrochemical detectors, but their ability to provide product identification and structural characterization is unrivalled. Current mass spectrometers can easily detect analytes in the nanomolar concentration range, while tandem mass spectrometry allows detailed structural analysis of sample ions of interest. The mass of an analyte gives specific information on the chemical identity; the higher the mass accuracy the easier the assignment to a particular chemical formula. Oxidation and reduction reactions often result in mass changes, for example by introduction of an oxygen atom, or by dehydrogenation. With tandem mass spectrometry the chemical structure can be elucidated further: analyte ions are fragmented, typically by impact with a collision gas (CID fragmentation). The mass of the resulting fragment ions can then be recorded, and using general chemical principles, the functional groups can often be confirmed or assigned. Alternatively, spectral libraries can be used to match fragment ion spectra, although this is not yet common practice for CID type MS/MS spectra due to the difficulty in standardizing fragmentation conditions.

In many cases electrochemical reactions can be done off-line and products analyzed separately by mass spectrometry, but the on-line coupling has some distinct advantages. First, it allows for an integrated, automated system, facilitating high-throughput applications. In addition, direct and time-resolved analysis of reaction products is possible, with a minor time delay dependent only on the dead volume between the working electrode and the mass analyzer. In an integrated system [5-7] it is even possible to observe reaction intermediates. Fig. **1** gives an overview of the different combinations of electrochemistry, liquid chromatography and mass spectrometry and some of their applications, which will be described in more detail in the next sections.

2. COUPLING OF ELECTROCHEMISTRY TO MASS SPECTROMETRY: CHALLENGES & SOLUTIONS

A major hurdle in the development of EC-MS in the early period was the interfacing of the electrochemical cell

*Address correspondence to this author at Mass Spectrometry Core Facility, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands; Fax: +31-50-363 8347; E-mail: h.p.permentier@rug.nl

1389-5575/08 $55.00+.00

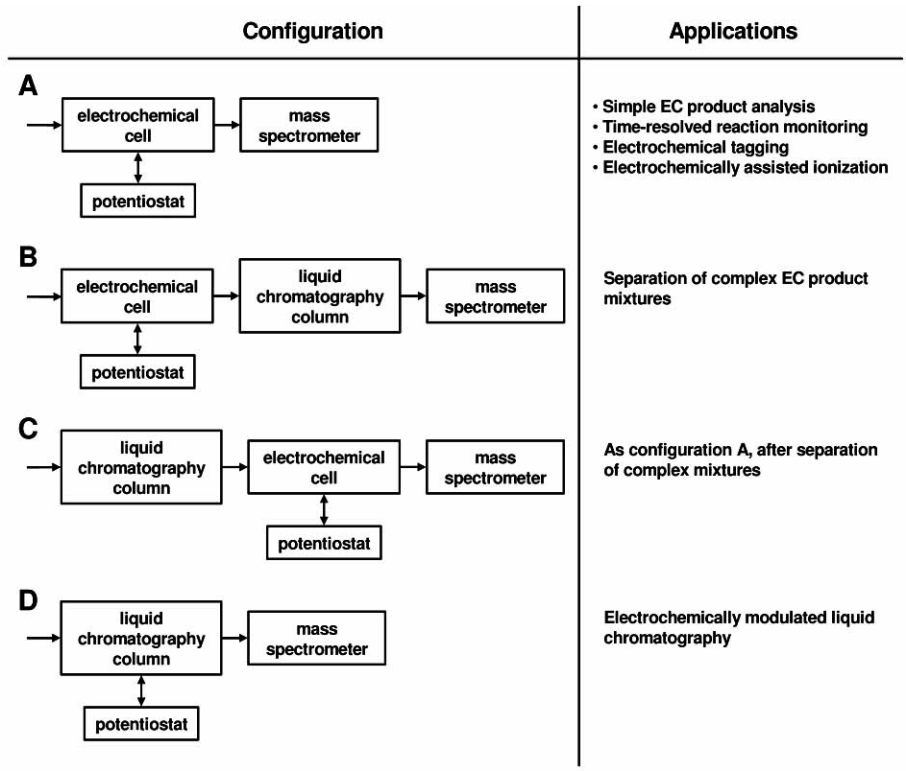

Fig. (1). Schematic view of configurations for combining electrochemistry, liquid chromatography and mass spectrometry, and their main applications.

with the ionization source of the mass spectrometer. Hambitzer and Brajter-Toth [1, 2, 8-11] used thermospray as the ionization method, but electrospray has become the ionization method of choice for (often fragile) biomolecules. Electrospray ionization (ESI) has the advantage that it is a soft ionization method: minimal fragmentation or decomposition of analytes occurs during the process. ESI releases ions from the liquid phase by applying a potential difference between a conducting electrospray emitter (typically a metal needle with an orifice diameter in the µm range, used with flow rates from the ml/min down to the sub-µl/min range) and the entrance of the mass analyzer (Fig. **2**). The emitter is generally at a relative potential of several kV above or below the mass analyzer, for positive or negative ionization, respectively. A potential applied to the emitter results in electrochemical processes in the emitter and liquid phase [12]. These inherent electrochemical reactions have been studied in detail, to gain a better understanding of the electrospray process, but they also may affect and modify the analytes that are subsequently detected by the mass spectrometer [13-17]. Under some conditions even chromatographic column material, like graphite, can act as an electrochemical electrode, when it forms a grounded loop with the electrospray emitter and the mass analyzer [18, 19]. For certain compounds that are very sensitive to oxidation (e.g., quinols), special care must be taken to avoid spurious oxidation [20, 21]. This effect can also be used in a positive manner, for modification and derivatization of proteins as will be discussed in section 4.4.

EC-MS methods most commonly employ a separate electrochemical cell that is linked on-line to an electrospray emitter. Commercial flow-through cells include thin-layer cell types and cells with porous working electrodes. In these set-ups care is taken to avoid as much as possible the influence of the electrospray potential, which in a normal ESI-MS setup produces a current through the liquid phase between the emitter and up-stream grounding points. Solutions for coupling EC to ESI-MS in various ways have been described by several groups, most notably by van Berkel and coworkers [6, 7, 22-26], but also by others [5, 27-29]. In many cases the cell and ESI emitter have become integrated, and the emitter is employed as an electrode in the circuit. This pre-

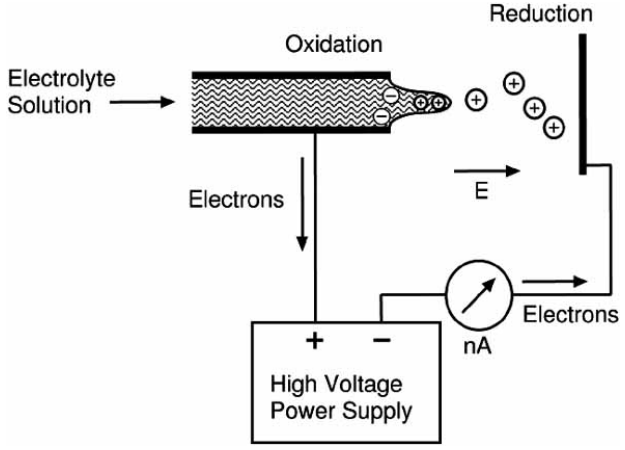

Fig. (2). Schematic view of an electrospray ionization source connected to a mass analyzer; electrical current direction is indicated for positive ionization mode.

vents additional, uncontrolled reactions in the emitter. Alternatively, the decoupling of the electrochemical cell and electrospray emitter is advantageous for the prevention of unexpected reactions: this allows for better control of the cell potential without interference from the electrospray potential.

The design, material and dimensions of the electrode have practical performance implications in EC-MS [22, 30]. Carbon-based working electrodes (typically in the form of glassy carbon) are most commonly used in organic electrochemistry, but have a strong tendency to foul by absorption of analytes, in particular large biomolecules like proteins. Various types of metals have also been employed for specific purposes, including gold, platinum and copper [31, 32]. Electrode surfaces can be modified with redox-active intermediates or other surface modifications, to both change the electrochemical behavior and diminish the extent of absorption. Section 3.2 discusses the use of electrodes with modified surfaces in the context of mimicking Cytochrome P450-mediated reactions.

An issue that emerges when EC is coupled to ESI-MS is signal suppression by the electrolyte components of the solvents used for EC. Buffer salts are desirable for conductivity in the electrochemical cell, but MS signals can be suppressed dramatically by even low millimolar salt concentrations. Salt ions compete with analytes for ion formation in electrospray. Their concentration can be reduced by dilution or by chromatographic separation in between EC and MS. Alternatively, volatile salts that have less suppression effects can be used. A compromise usually has to be made to accommodate both techniques.

Other EC-MS techniques which are not further discussed here, are EC interfaced with inductively coupled plasma-MS, a technique for trace metal analysis [33, 34], and Differential Electrochemical Mass Spectrometry (DEMS), which is used to analyze volatile products of electrochemical reactions, such as CO, directly upon their formation [35]. In DEMS experiments products are directly introduced through a membrane or pinhole into the vacuum of the mass spectrometer and ionized by electron impact.

3. EC-MS IN DRUG METABOLITE & ENZYME RESEARCH

A major application of EC-MS is the study of oxidation reactions and products of drugs and xenobiotics and the relationship of these reactions to *in vivo* metabolism. In this section, the focus will be on drug metabolism in relation to Cytochrome P450 (CYP450) reactions, with several other applications of EC-MS listed in section 3.3.

3.1. Cytochrome P450 Oxidation Reactions & EC Mimicking

Cytochrome P450-type monooxygenase enzymes are important biotransformers of xenobiotic compounds, including both drugs and environmental agents. Their action constitutes an important stage of phase I drug metabolism, aimed at transforming the targeted compounds into more polar derivatives. A unique feature of CYP450 enzymes is their broad range of substrates; the reactivity can be categorized in two types: single electron transfer (SET) oxidation reactions, and hydrogen atom transfer/abstraction (HAT) type reactions. The range of reaction types and their action on different functional groups is summarized in Table **1** and Fig. **3**. Recent reviews of Cytochrome P450 oxidation reactions can be found in [36] and [37]. A number of CYP450 isozymes are known, that differ in reaction product ratios, such as the ratio of hydroxylation versus epoxidation, and different isozymes often work in concert *in vivo*.

The study of drug metabolism is of critical importance in drug development. Metabolic conversion is often studied in liver preparations, or microsomes, which contain the enzymes in their natural environment, allowing the full range of metabolic products to be formed. This method has the disadvantage that products have to be isolated from a rather complex mixture. Mimicking CYP450 reactions *in vitro* could be of great use, since it allows for faster and easier product analysis and therefore larger scale screening methods. In addition, it allows the production of metabolites in sufficient purity and quantity for use as standards in subsequent *in vivo* studies. A number of *in vitro* methods to use CYP450 enzymes [38] or CYP450- type reactions by biomimetic catalysts such as metalloporphyrins [39] have been investigated. Electrochemistry can also play a very useful role in CYP450-catalyzed drug metabolism studies. The combination of EC-MS allows for rapid and detailed screening of reaction products. The major challenge is to find a system that replicates the native CYP450 reactions as closely as possible without compromising experimental through-put.

Direct electrochemistry of CYP450 has been investigated with the enzyme absorbed on a pyrolytic graphite electrode [40]; its redox properties, but no enzymatic conversions were studied. When the enzyme is attached to the electrode, electrochemistry can be used to regenerate the cofactor [38]. To further reduce the complexity of the system only the heme cofactor can be used; either added to the liquid phase during passage through the electrochemical cell or bound to the electrode (see also section 3.2). Different porphyrins or metal ligands can be used to alter the reactivity.

Experiments with unmodified electrodes have been performed by Jurva *et al*. [41, 42], and Nozaki *et al*. [43]. Glassy carbon electrodes are suitable for mimicking the SET type, but not the HAT type reactions, presumably because the iron-oxygen intermediate present in CYP450 cannot be mimicked directly by electrochemistry. A different approach for extending the range of reaction types is by use of the Fenton reaction to produce hydroxyl radicals by reaction of ferrous iron with hydrogen peroxide [44]. The electrochemical cell reduces Fe^{3+} to Fe^{2+} and thereby initiates hydroxyl radical formation and its reaction with analytes. With the Fenton reaction, several different types of reaction products, not previously found with direct electrochemical conversion, were observed. In a recent comprehensive study Johansson *et al*. [45] have considerably expanded the range of successfully mimicked reaction types by performing electrochemistry at different solvent pH levels, and by the use of the electrochemical Fenton system. The electrochemical reactions of a range of drug compounds with a variety of functional groups were compared with the observed reactions of the same molecules in liver microsomes. The conversion reac-

Table 1. Cytochrome P450 Reaction Types, Categorized by (Major) Reaction Mechanism, and the Electrochemical Methods Used for Mimicking them

Reaction Mechanism	Electrochemical Method	Reference
Single electron transfer		
Epoxidation	glassy carbon	[42]
Alcohol oxidation	glassy carbon	[42]
Aldehyde oxidation	no reaction	
N-oxidation	EC-induced Fenton reaction	[45]
S-oxidation	glassy carbon; EC-induced Fenton reaction	[42, 45]
P-oxidation	glassy carbon	[42]
Deamination	no reaction	
Dehalogenation	glassy carbon	[42]
Single electron transfer and/or hydrogen atom transfer		
Dehydrogenation	glassy carbon, acidic conditions; EC-induced Fenton reaction	[42, 45]
N-dealkylation	glassy carbon, basic and acidic; EC-induced Fenton reaction	[42, 45]
Hydrogen atom transfer		
Aromatic and aliphatic hydroxylation	EC-induced Fenton reaction	[44, 45]
O-dealkylation	glassy carbon, acidic; EC-induced Fenton reaction	[45]

tions achieved with different electrochemical methods are summarized in Table **1**.

During *in vivo* metabolism the direct oxidative reactions of phase I metabolism are followed by conjugate-formation in phase II metabolism. Electrochemical mimicking of both steps in a single experiment has been explored already in the very first EC-MS studies of CYP450 [46], which involved coupling of paracetamol to glutathione. Recently two groups [47, 48] have shown similar results for paracetamol reactions with glutathione and N-acetylcysteine.

The products observed by EC-MS show that there are strong parallels between the reactions performed by CYP450 and electrochemical reactions under appropriate experimental conditions. Because of current limitations in the exact replication of the active site of CYP450, in particular the iron-oxygen intermediate, the full range of reaction types cannot yet be mimicked. Modification of electrode material and surface is the most promising approach towards further improvement and extension of CYP450-like electrochemical reactions.

3.2. Improving Cytochrome P450 Mimicking with Modified Electrodes

Electrochemistry of proteins on electrodes is extensively studied in relation to their use in biosensors [49, 50]. Enzyme-modified electrodes are used to sense the presence of the appropriate substrate. CYP450 can also be readily bound to electrode surfaces [40, 51]. Alternatively, the reactive heme group can be bound to the electrode without the surrounding protein. The protein environment of the active site is most likely responsible for distinctive or preferred reactions of the different isoforms of CYP450 [52], but modification of the porphyrin environment on electrodes to reflect this is not feasible. Different porphyrin types and analogs, and different metallic centers can be employed to tune the redox reactions [53], but not the steric effects of the surrounding protein. The way porphyrin is immobilized may well influence its reactivity. For example, the use of a thiolate ligand to form a coordinate bond with the metal center, which emulates the binding of cysteine in CYP450, promotes hydrogen abstraction reactions [54].

Porphyrins can be used in solution, but in an on-line system it is more effective and convenient to immobilize them on the electrode. Gold and silver electrodes can be readily covered with self-assembled monolayers (SAM) of thiolated compounds. The latter can have various functional groups to bind a secondary layer of compounds, in this case porphyrins. Proteins can also be bound to a SAM with appropriate functional groups. For example, cytochrome *c* and CYP450 can be bound to mercaptoundecanoic acid by ionic interac-

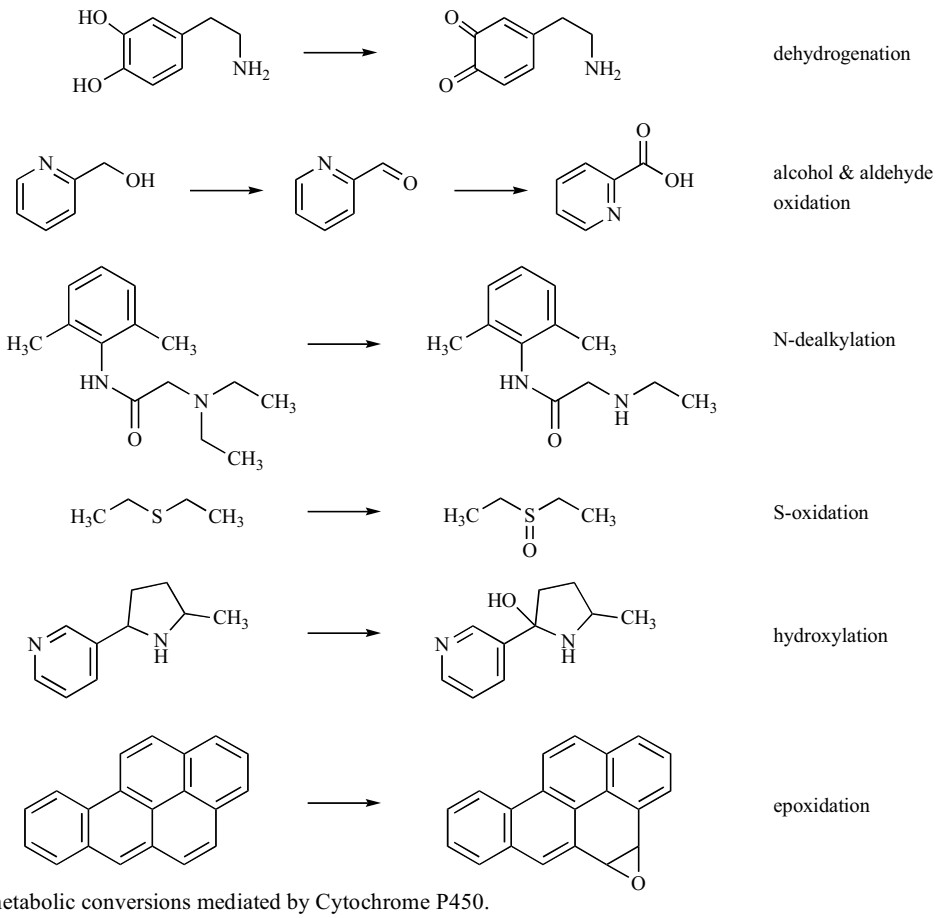

Fig. (3). Types of metabolic conversions mediated by Cytochrome P450.

tion [51]. These enzymes were also successfully bound to a clay-modified carbon electrode [51]. Direct electrochemistry of CYP450 embedded in a surfactant film on a carbon electrode has been shown by Shukla *et al*. [40]. An extensive overview of methods for binding enzymes to various electrode materials is given by Mueller [49].

3.3. Other EC-MS Applications in (Drug) Metabolism

Table **2** gives an overview of the EC-MS applications that have been published for electrochemical conversion of either drugs or xenobiotic compounds, other than those referred to in section 3.1. In most cases the compounds were

Table 2. EC-MS Applications in Metabolism of Drugs and Xenobiotics

Compound	Reaction Type	Method Remarks	Reference
N,N-dimethylaniline	dimerization	platinum electrode	[1]
dopamine	dehydrogenation	(glassy) carbon electrode	[9, 23]
reserpine	N-oxidation, dehydrogenation	glassy carbon electrode, various new cell set-ups	[6, 7, 25, 26]
olsalazine	dehydrogenation	glassy carbon electrode, new thin layer cell set-up	[28]
(*p*-chlorophenyl)aniline	dimerization	glassy carbon electrode, detection in rat bile & urine, with LC-EC-MS	[55]
paracetamol	dehydrogenation, subsequent reaction with glutathione	glassy carbon electrode	[46-48]
zotepine, chlorpromazine	N-dealkylation, S-oxidation	glassy carbon electrode	[43]

used in pure form, to test the efficiency of particular EC cell set-ups. The recent paper of Chen *et al*. [55] described a possible clinical application, where (*p*-chlorophenyl)aniline oxidation products are determined in rat bile and urine. Gamache *et al*. [56] have described an electrochemical array system, consisting of several electrochemical cells in series, coupled on-line or in parallel with MS. Both electrochemical and MS detection, and electrochemical oxidation detected with MS, provide a rich set of data for metabolomic studies, as shown with experiments on rat urine.

4. EC-MS IN PROTEIN ANALYSIS

4.1. Protein Oxidation and Electrochemistry Overview

Oxidation of proteins is a subject of great clinical importance since it is associated with a wide variety of disease processes and with ageing [57]. Reactive agents, typically reactive oxygen species (ROS), can be derived from both the environment and from metabolic processes, in particular from the activity of peroxidases [58]. Several side chains of naturally occurring amino acids are susceptible to direct oxidation or reduction, notably the sulfur-containing cysteine and methionine, and the aromatic tyrosine and tryptophan side chains. Their most common electrochemical reaction products are show in Fig. **4**. Radical intermediates, which may be generated *in vivo* by peroxidases, can also react with other amino acids, such as phenylalanine.

Redox reactions are extremely important in view of protein function. In redox-active enzymes, cofactors are commonly required, but amino acid side chains may also be involved in electron transfer reactions. Electrochemistry has long been used to detect and study redox properties of proteins. Electrodes can be used as artificial electron donors or acceptors; the current is a measure of enzyme activity. In this configuration an electrochemical cell may function as a biosensor for enzyme activity. In effect, the conversion is still fully enzymatic and the electrochemical cell serves for detection purposes only. Enzyme products can also be detected on-line with a mass spectrometer.

Induction of oxidation reactions in proteins has similar applications as oxidation of drug molecules, discussed in the previous section. The effect of (some) *in vivo* oxidation reactions can be mimicked in a controlled way, which allows a more detailed study of both reaction mechanisms and products. Oxidation by chemical, photochemical and electrochemical methods gives rise to similar products, although method-specific products may also be generated. Electrochemically generated reactive species, typically radicals, can react with a protein, but alternatively, direct electrochemical oxidation of susceptible moieties in a protein at the electrode surface can occur. Both reaction types may be used for the qualitative or quantitative analysis of a protein.

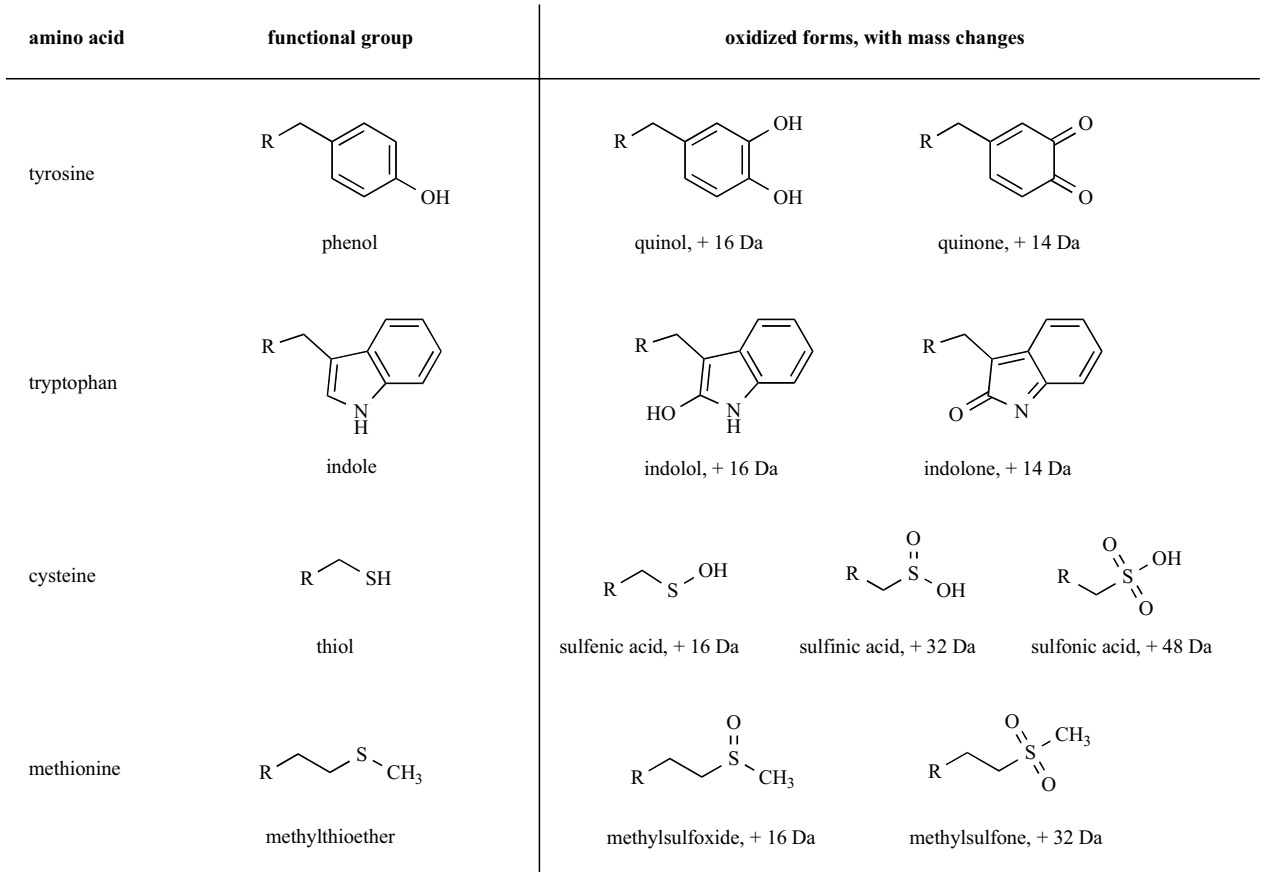

Fig. (4). Electrochemically oxidizable amino acids, functional groups and oxidation products with associated mass changes.

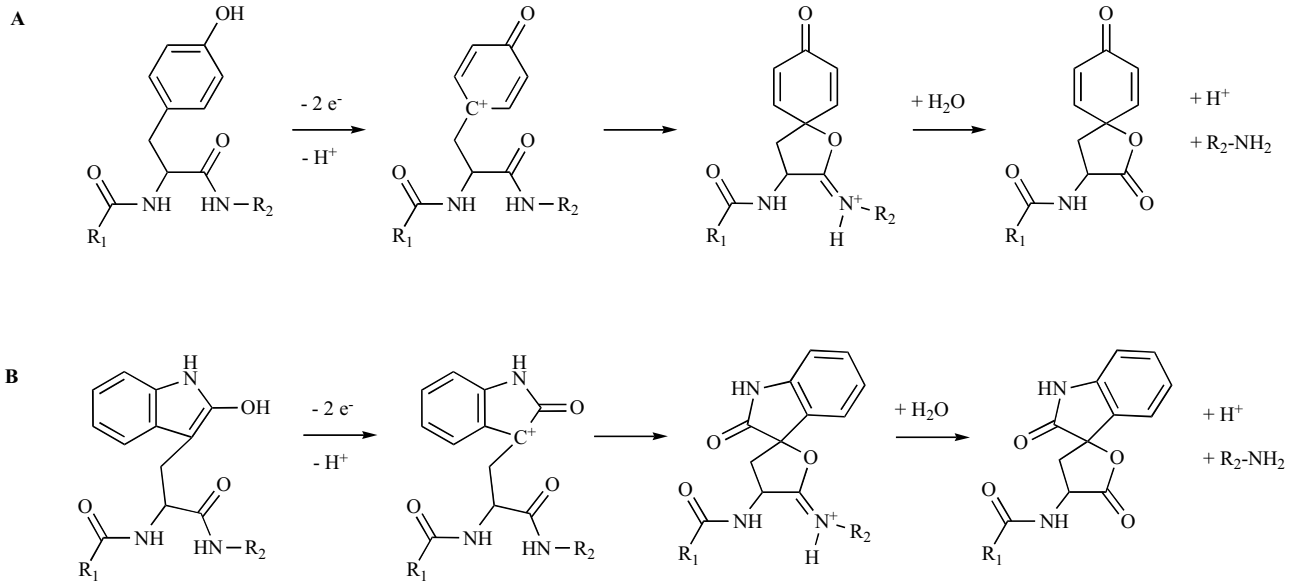

Fig. (5). Reaction pathway of peptide cleavage at tyrosine (A) and tryptophan (B). Tryptophan is first oxidized to 1-hydroxytryptophan.

4.2. Study of Redox-(Metallo)Proteins

Electrochemical study of proteins has largely focused on their redox-active groups, in particular metal ions and metal-binding cofactors (e.g., heme in CYP450 described in section 3, [40]). Redox activity of metalloproteins can occur in an electrochemical cell, but also in the electrospray emitter. On-line EC-MS experiments by Johnson *et al*. [59] have resulted in the successful reduction of several types of redox enzymes, namely cytochrome *c* with an iron-heme as the cofactor, superoxide reductase with a non-heme iron ion, ferredoxin, with a manganese substituted iron-sulfur center, and azurin, with a copper ion cofactor. The oxidation state of a protein can be inferred from its charge state, which is measured by the mass spectrometer. A general review of mass spectrometric analysis of metalloproteins was published recently by Kaltashov *et al*. [60].

4.3. Protein Side-Chain Modification: Mimicking *In Vivo* Oxidation

Oxidative modification of protein side-chains of both redox and non-redox proteins by electrochemistry has not received much attention up to date. The electrochemical behavior of peptides, and particularly amino acids, however, has been studied extensively, but mainly in the context of electrochemical detection. Analysis of the specific modifications of peptides and proteins proved difficult before the advent of suitable mass spectrometric techniques. Large scale experiments that allow analysis of oxidative modifications on a proteome-wide scale can now be performed. The modifications that are produced *in vivo* are attractive biomarker targets, possibly facilitating early diagnosis of a number of diseases [57, 61, 62]. Peroxynitrite is a reactive nitrogen species (RNS) that may be formed from NO by peroxidase activity and can give rise to nitration products. Tyrosine nitration is an indicator of the presence of RNS in a biological system, but may also have a cellular signaling function [63].

Brabec and coworkers were among the first to report irreversible electrooxidation in a protein: tyrosine and tryptophan were found to be oxidized in addition to heme in cytochrome *c* [64, 65]. Walton and coworkers discovered several unexpected reaction products of lysozyme upon oxidation at a copper electrode. Tyrosine nitration was observed, although no nitrogen source was added to the medium [66]. With a carbon electrode, nitration did not occur, but instead, methionine oxidation and cleavage of the peptide bond between two tryptophan residues were detected [67, 68]. The latter observation was the first reported electrochemical cleavage of a native protein, which is discussed in more detail in section 4.6. Tyrosine nitration in lysozyme has recently been studied in more detail by mass spectrometry [69], with the aim of developing a preparative method. Carefully controlled conditions allowed specific nitration using a platinum electrode in the presence of nitrite.

Mass spectrometry has also been employed to detect amino acid modifications in metalloproteins. Modification by metal-catalyzed oxidation has been observed and used to pinpoint the metal-binding sites of metalloproteins [70, 71].

4.4. On-Line Protein Derivatization by Electrospray

Electrochemical tagging of cysteine residues is an innovative method that makes use of the potentially troublesome electrochemical processes in the electrospray emitter. This technique was developed by Girault and coworkers [72-76]. Cysteine residues in proteins, due to their relative scarcity and their reactivity, are attractive targets for chemical derivatization in proteomics studies, for example for relative protein quantification by differential labeling with heavy and light-isotope coded reagents. The reactivity of the thiols is exploited in the electrotagging method: a tagging agent such as *p*-hydroquinone is oxidized electrochemically during nanoelectrospray ionization and subsequently reacts with cysteine residues (Fig. **6A**). The specificity of this reaction allows it to be used as an on-line method for chemical de-

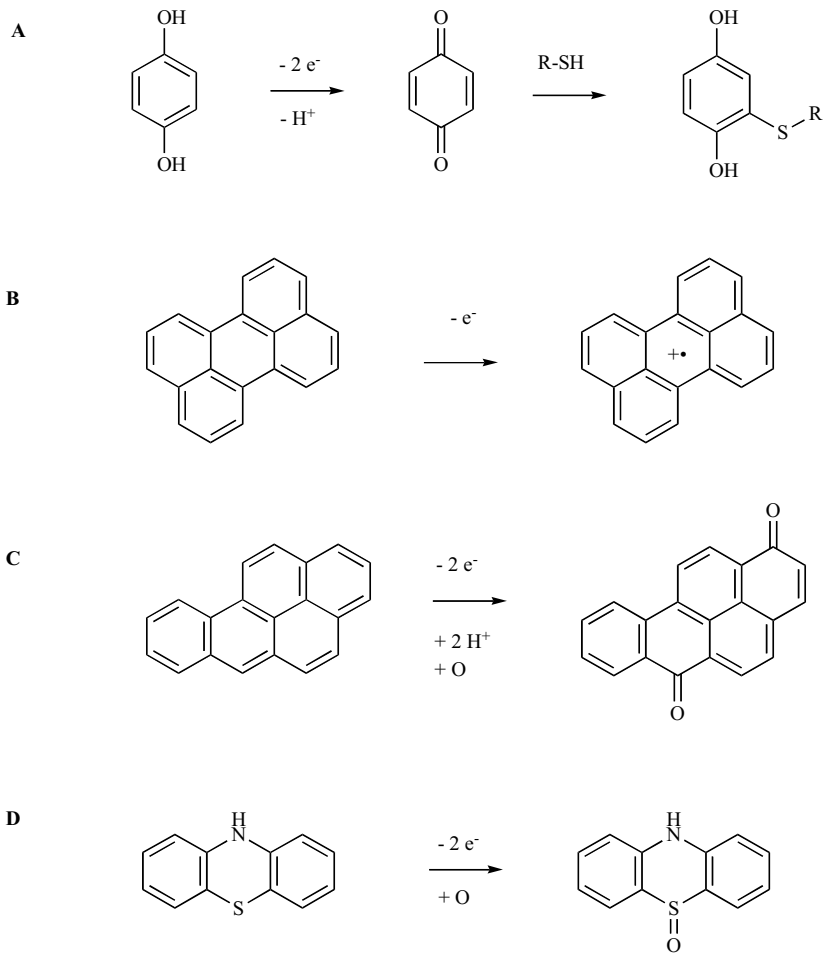

Fig. (6). A. Electrochemical tagging reaction of the thiol-group of a cysteine residue with hydroquinone. B-D. Electrochemical reactions of analytes to more readily ionized products: perylene (B), benzopyrene (C), phenothiazine (D).

rivatization. An advantage is that the derivatization takes place immediately after separation of the peptide targets, and before mass analysis, circumventing chromatography problems of modified peptides. Van Berkel *et al*. [24] have shown that this method is also able to reduce disulfide bonds concurrent with derivatization.

4.5. Protein Structure Analysis and Foot Printing

An application of electrochemical oxidation that is unique for proteins is tertiary and quaternary structure analysis. In this method, also called footprinting, the parts of the proteins that are exposed to the environment are tagged by reactive species or directly modified [65, 77-79]. Reactive species are added to a protein mixture or generated by photochemical or electrochemical means. In the latter case, reaction takes place either directly at the electrode surface or by way of radical intermediates formed e.g. by the Fenton reaction. Oxidation in the electrospray emitter has also been employed [80]. Subsequent analysis of oxidized proteins or protein complexes (e.g., by analysis of a protein digest with LC-MS/MS) is used to pinpoint the locations of the oxidized amino acids. These can be interpreted as being exposed on the surface of the protein, and used to construct or verify a tertiary or quaternary structural protein model, or to determine the interface region of two interacting proteins [81]. Further development of mass spectrometric instruments and methods for intact protein fragmentation (often called top-down analysis) [82], will allow a completely on-line system for modification and analysis.

4.6. Oxidative Protein Cleavage

In the rapidly growing field of proteomics, identification and characterization of proteins by mass spectrometry requires their cleavage into smaller peptides. Protein cleavage can be achieved by chemical and enzymatic means. The latter is by far the most common, because of its ease and specificity. Electrochemical oxidative cleavage at specific residues in peptides has been described more than 40 years ago [83-86], but has not received much attention since. More recently, it has been observed coincidentally by Walton and coworkers [67] in lysozyme (see section 4.3). Around the same time MacDonald *et al*. [87] reported oxidative cleavage of tryptophanyl and tyrosyl-glycine bonds on a platinum electrode. Electrochemical oxidation and cleavage at the C-terminal side of tyrosine and tryptophan residues in peptides [88] and proteins [89] has been studied more fully recently. An on-line EC-MS or EC-LC-MS system was used to detect and characterize the cleavage products and non-cleavage

oxidation products. The reaction mechanisms have not been studied in much detail, but judged from the products, they are probably similar to analogous chemical oxidation reactions (Fig. **5**) [90, 91]. An interesting observation was the resistance to electrochemical oxidation of phosphorylated tyrosine residues: phosphorylation blocks the tyrosine hydroxyl group, and no reaction products were observed upon oxidation [88]. This behavior could be used as an alternative way to distinguish between phosphorylated and unphosphorylated tyrosine residues in proteins. Tyrosine phosphorylation is a very important regulatory mechanism in protein activity, and its detection is a major issue in proteomic research [92]. EC-MS could add to the repertoire of methods.

5. ENHANCEMENT OF MASS SPECTROMETRIC DETECTION THROUGH ELECTROCHEMISTRY

5.1. Electrochemically Assisted Ionization

Electrochemical modification of analytes can be employed to improve their ionization efficiency in electrospray. In general, ionization of compounds with electrospray relies on the formation of ionized species in solution; for positive ions typically by protonation or by metal-ion adduct formation. The ease with which compounds can be ionized therefore depends on suitable functional groups for protonation or adduct formation. When these groups are not available, electrochemical modification can create them in a process called Electrochemically Assisted Ionization (EAI). The conversion (see Fig. **6B-D**) can be direct, as shown for the generation of a radical cation of the polycyclic aromatic compound perylene [22] or for triphenylamine [93]. The conversion of dibutylsulfide into dibutylsulfoxide, benzopyrene into benzopyrene quinone [42], or phenothiazine into phenothiazine sulfoxide [94] involves introduction of oxygen. These oxygenated products can then be readily protonated and ionized by electrospray. Alternatively, the compound of interest can be derivatized first with an electroactive group. A common choice for this purpose is ferrocene in which Fe^{2+} is oxidized to Fe^{3+} to give the ferrocenium ion [95]. Derivatization of non-polar alcohols with ferrocene analogs has been the main application [7, 95, 96]. The stable, preformed ferrocenium ions increase sensitivity in mass spectrometric analysis, enhancing sensitivity even for compounds that can normally be protonated. A related method was described by Rohner *et al*. [97], where a sacrificial electrode was used to generate metal ions that could subsequently form complexes with peptides.

5.2. Electrochemically Modulated Liquid Chromatography

The working electrode of a flow-through electrochemical cell can retain analytes by physical absorption, the extent of which can be modulated by the applied potential. This absorption effect, which is undesirable in most electrochemical applications, can be employed to achieve electrochemically modulated liquid chromatography (EMLC), where a potential applied to the stationary phase material, such as porous graphite, is used to absorb and release analytes. As noted in section 2, inadvertent electrochemical reactions can already occur on-column if the column is not properly uncoupled from the electrospray emitter [18, 19]. EMLC can be used to preconcentrate analytes on the column and in this way achieve better separation, allow for efficient sample desalting, and improve detection through sample transformation (see section 5.1). The concurrent use of concentration and oxidation to generate and separate products alleviates the need for a separate cell and LC column. In EMLC, analytes are not eluted by changes in the mobile phase, but by changing the potential applied to the column [98]. Deng *et al*. [99], and Pretty *et al*. [100] have shown separation of mixtures of corticosteroids, benzodiazepines, and tamoxifens, using EMLC in this way. Preconcentration without a column is also possible. Bökman *et al*. [28] have described preconcentration and desalting of thiolated compounds on a gold electrode. EMLC has also been used to achieve protein capture and separation on a microdevice [101, 102].

6. FUTURE PERSPECTIVES

Miniaturization of both electrochemical cells and separation methods is currently a hot topic. Electrochemical techniques are very suitable for chip applications (see [103] for a recent overview). Mengeaud *et al*. [104] have produced a ceramic electrochemical microreactor that can be coupled on-line with MS. Automation is another area where progress is being made. A robotic system for electrochemical arrays has been described by Erichsen *et al*. [105]. A system with multiple electrochemical cells in series as described by Gamache *et al*. [56] is another way to increase the amount of information generated in a single analysis of a complex sample. Integration of an electrochemical cell and an electrospray emitter on the same device solves some of the interfacing problems of EC and MS and eliminates the delay time of a separate electrochemical cell [7, 24]. Inclusion of an LC column for product separation is also feasible, creating a veritable lab-on-a-chip system. Integrated LC-ESI chips are already commercially available. Easily exchangeable or even disposable EC-LC-ESI devices could increase the flexibility for the researcher, since they would allow for the optimal choice of electrode or LC system depending on the types of analyte. EMLC (see section 5.6) has also been shown to be well suited for miniaturization [101, 102].

In summary, electrochemistry is a powerful and versatile tool to study small molecules, but also proteins. Besides its well-known use for electrochemical detection, it is possible to generate oxidative modifications that mimic *in vivo* drug metabolism, and native protein damage or metabolism. Applications specific for proteins are tertiary structure analysis, and protein processing for proteomic applications. In all cases, information can be obtained from the electrochemical data, with mass spectrometry as a very powerful complementary technique that allows detailed structural analysis of products. When the electrochemical cell is coupled on-line to mass spectrometry, product analysis can be done quickly, in real-time and in an automatic fashion, which facilitates high-throughput applications.

REFERENCES

[1] Hambitzer, G.; Heitbaum, J. *Anal. Chem.,* **1986**, *58*, 1067.
[2] Volk, K. J.; Yost, R. A.; Brajter-Toth, A. *Anal. Chem.,* **1989**, *61*, 1709.
[3] Van Berkel, G. J. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1691.
[4] Diehl, G.; Karst, U. *Anal. Bioanal. Chem.,* **2002**, *373*, 390.
[5] Xu, X.; Lu, W.; Cole, R. B. *Anal. Chem.,* **1996**, *68*, 4244.

[6] Van Berkel, G. J.; Asano, K. G.; Granger, M. C. *Anal. Chem.,* **2004**, *76*, 1493.
[7] Van Berkel, G. J.; Kertesz, V.; Ford, M. J.; Granger, M. C. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1755.
[8] Hambitzer, G.; Heitbaum, J.; Stassen, I. *Anal. Chem.,* **1998**, *70*, 838.
[9] Regino, M. C. S.; Brajter-Toth, A. *Anal. Chem.,* **1997**, *69*, 5067.
[10] Regino, M.; Weston, C.; Brajter-Toth, A. *Anal. Chim. Acta,* **1998**, *369*, 253.
[11] Volk, K. J.; Yost, R. A.; Brajter-Toth, A. *Anal. Chem.,* **1992**, *64*, A21.
[12] Van Berkel, G. J.; Zhou, F. *Anal. Chem.,* **1995**, *67*, 2916.
[13] Fernandez de la Mora, J.; Van Berkel, G. J.; Enke, C. G.; Cole, R. B.; Martinez-Sanchez, M.; Fenn, J. B. *J. Mass Spectrom.,* **2000**, *35*, 939.
[14] Van Berkel, G. J.; Asano, K. G.; Schnier, P. D. *J. Am. Soc. Mass Spectrom.,* **2001**, *12*, 853.
[15] Konermann, L.; Silva, E. A.; Sogbein, O. F. *Anal. Chem.,* **2001**, *73*, 4836.
[16] Rohner, T. C.; Lion, N.; Girault, H. H. *Phys. Chem. Chem. Phys.,* **2004**, *6*, 3056.
[17] Ochran, R. A.; Konermann, L. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1748.
[18] Liu, S.; Griffiths, W. J.; Sjövall, J. *Anal. Chem.,* **2003**, *75*, 1022.
[19] Törnkvist, A.; Nilsson, S.; Amirkhani, A.; Nyholm, L. M.; Nyholm, L. *J. Mass Spectrom.,* **2004**, *39*, 216.
[20] Kertesz, V.; Van Berkel, G. J. *J. Mass Spectrom.,* **2001**, *36*, 204.
[21] Smith, A. D.; Moini, M. *Anal. Chem.,* **2001**, *73*, 240.
[22] Zhou, F.; Van Berkel, G. J. *Anal. Chem.,* **1995**, *67*, 3643.
[23] Deng, H.; Van Berkel, G. J. *Electroanalysis,* **1999**, *11*, 857.
[24] Van Berkel, G. J.; Kertesz, V. *Anal. Chem.,* **2005**, *77*, 8041.
[25] Kertesz, V.; Van Berkel, G. J.; Granger, M. C. *Anal. Chem.,* **2005**, *77*, 4366.
[26] Kertesz, V.; Van Berkel, G. J. *J. Am. Soc. Mass Spectrom.,* **2006**, *17*, 953.
[27] Bond, A. M.; Colton, R.; D'Agostino, A.; Downard, A. J.; Traeger, J. C. *Anal. Chem.,* **1995**, *67*, 1691.
[28] Bökman, C. F.; Zettersten, C.; Sjöberg, P. J. R.; Nyholm, L. *Anal. Chem.,* **2004**, *76*, 2017.
[29] Zettersten, C.; Lomoth, R.; Hammarström, L.; Sjöberg, P. J. R.; Nyholm, L. *J. Electroanal. Chem.,* **2006**, *590*, 90.
[30] Jurva, U. *Electrochemistry on-line with mass spectrometry - Instrumental methods for in vitro generation and detection of drug metabolites.* Doctoral thesis, University of Groningen, The Netherlands, **2004**.
[31] Lund H.; Hammerich O. *Organic Electrochemistry*, Marcel Dekker: New York, **2001**.
[32] Lund, H. *J. Electrochem. Soc.,* **2002**, *149*, L8.
[33] Zhou, F.; Johnston, M. V. *Electrophoresis,* **2005**, *26*, 1383.
[34] Liljegren, G.; Forsgard, N.; Zettersten, C.; Pettersson, J.; Svedberg, M.; Herranen, M.; Nyholm, L. *Analyst,* **2005**, *130*, 1358.
[35] Baltruschat, H. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1693.
[36] Meunier, B.; de Visser, S. P.; Shaik, S. *Chem. Rev.,* **2004**, *104*, 3947.
[37] Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.,* **2005**, *105*, 2253.
[38] Chefson, A.; Auclair, K. *Mol. BioSyst.,* **2006**, *2*, 462.
[39] dos Santos, M. D.; Martins, P. R.; dos Santos, P. A.; Bortocan, R.; Iamamoto, Y.; Lopes, N. P. *Eur. J. Pharm. Sci.,* **2005**, *26*, 62.
[40] Shukla, A.; Gillam, E. M.; Mitchell, D. J.; Bernhardt, P. V. *Electrochem. Comm.,* **2005**, *7*, 437.
[41] Jurva, U.; Wikström, H. V.; Bruins, A. P. *Rapid Comm. Mass Spectrom.,* **2000**, *14*, 529.
[42] Jurva, U.; Wikström, H. V.; Weidolf, L.; Bruins, A. P. *Rapid Comm. Mass Spectrom.,* **2003**, *17*, 800.
[43] Nozaki, K.; Kitagawa, H.; Kimura, S.; Kagayama, A.; Arakawa, R. *J. Mass Spectrom.,* **2006**, *41*, 606.
[44] Jurva, U.; Wikström, H. V.; Bruins, A. P. *Rapid Comm. Mass Spectrom.,* **2002**, *16*, 1934.
[45] Johansson, T.; Weidolf, L.; Jurva, U. *Rapid Comm. Mass Spectrom.,* **2007**, *21*, 2323.
[46] Getek, T. A.; Korfmacher, W. A.; McRae, T. A.; Hinson, J. A. *J. Chromatogr.,* **1989**, *474*, 245.
[47] Lohmann, W.; Karst, U. *Anal. Bioanal. Chem.,* **2006**, *386*, 1701.
[48] Madsen, K.; Olsen J.; Hansen, S.; Jurva, U. *Chem. Res. Tox.,* **2007**, *20*, 821.
[49] Mueller, A. *Mini Rev. Med. Chem.,* **2005**, *5*, 231.
[50] Bakker, E.; Qin, Y. *Anal. Chem.,* **2006**, *78*, 3965.
[51] Scheller, F. W.; Wollenberger, U.; Lei, C.; Jin, W.; Ge, B.; Lehmann, C.; Lisdat, F.; Fridman, V. *Rev. Mol. Biotechnol.,* **2002**, *82*, 411.
[52] de Groot, M. J. *Drug Discov. Today,* **2006**, *11*, 601.
[53] Balogh, G. T.; Keserü, G. M. *Arkivoc,* **2004**, *7*, 124.
[54] Bukowski, M. R.; Koehntop, K. D.; Stubna, A.; Bominaar, E. L.; Halfen, J. A.; Münck, E.; Nam, W.; Que Jr, L. *Science,* **2005**, *310*, 1000.
[55] Chen, H.; Zhang, Y.; Mutlib, A. E.; Zhong, M. *Anal. Chem.,* **2006**, *78*, 2413.
[56] Gamache, P. H.; Meyer, D. F.; Granger, M. C.; Acworth, I. N. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1717.
[57] Dalle-Donne I.; Scaloni A. *Redox Proteomics: From Protein Modifications to Cellular Dysfunction and Diseases*, John Wiley & Sons: Hoboken, NJ, USA, **2006**.
[58] Imlay, J. A. *Annu. Rev. Microbiol.,* **2003**, *57*, 395.
[59] Johnson, K. A.; Shira, B. A.; Anderson, J. L.; Amster, I. J. *Anal. Chem.,* **2001**, *73*, 803.
[60] Kaltashov, I. A.; Zhang, M.; Eyles, S. J.; Abzalimov, R. R. *Anal. Bioanal. Chem.,* **2006**, *386*, 472.
[61] Rabilloud, T.; Chevallet, M.; Luche, S.; Leize-Wagner, E. *Expert Rev. Proteomics,* **2005**, *2*, 949.
[62] Dalle-Donne, I.; Scaloni, A.; Giustarini, D.; Cavarra, E.; Tell, G.; Lungarella, G.; Colombo, R.; Rossi, R.; Milzani, A. *Mass Spectrom. Rev.,* **2005**, *24*, 55.
[63] Alvarez, B.; Radi, R. *Amino Acids,* **2003**, *25*, 295.
[64] Brabec, V.; Mornstein, V. *Biophys. Chem.,* **1980**, *12*, 159.
[65] Brabec, V.; Mornstein, V. *Biochim. Biophys. Acta,* **1980**, *625*, 43.
[66] Richards, P. G.; Coles, B.; Heptinstall, J.; Walton, D. J. *Enzyme Microb. Technol.,* **1994**, *16*, 795.
[67] Walton, D. J.; Campbell, C. J.; Richards, P. G.; Heptinstall, J. *Electrochim. Acta,* **1997**, *42*, 3499.
[68] Kendall, G.; Cooper, H. J.; Heptinstall, J.; Derrick, P. J.; Walton, D. J.; Peterson, I. R. *Arch. Biochem. Biophys.,* **2001**, *392*, 169.
[69] Matters, D.; Cooper, H. J.; McDonnell, L.; Iniesta, J.; Heptinstall, J.; Derrick, P.; Walton, D.; Peterson, I. *Anal. Biochem.,* **2006**, *356*, 171.
[70] Bridgewater, J. D.; Vachet, R. W. *Anal. Biochem.,* **2005**, *341*, 122.
[71] Bridgewater, J. D.; Lim, J.; Vachet, R. W. *J. Am. Soc. Mass Spectrom.,* **2006**, *17*, 1552.
[72] Rohner, T. C.; Rossier, J. S.; Girault, H. H. *Electrochem. Comm.,* **2002**, *4*, 695.
[73] Roussel, C.; Dayon, L.; Lion, N.; Rohner, T. C.; Josserand, J.; Rossier, J. S.; Jensen, H.; Girault, H. H. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1767.
[74] Roussel, C.; Dayon, L.; Jensen, H.; Girault, H. H. *J. Electroanal. Chem.,* **2004**, *570*, 187.
[75] Dayon, L.; Roussel, C.; Prudent, M.; Lion, N.; Girault, H. H. *Electrophoresis,* **2005**, *26*, 238.
[76] Dayon, L.; Roussel, C.; Girault, H. H. *J. Proteome Res.,* **2006**, *5*, 793.
[77] Brabec, V.; Bianco, P.; Haladjian, J. *Biophys. Chem.,* **1982**, *16*, 51.
[78] Maleknia, S. D.; Downard, K. *Mass Spectrom. Rev.,* **2001**, *20*, 388.
[79] Guan, J. Q.; Chance, M. R. *Trends Biochem. Sci.,* **2005**, *30*, 583.
[80] Maleknia, S. D.; Chance, M. R.; Downard, K. M. *Rapid Comm. Mass Spectrom.,* **1999**, *13*, 2352.
[81] Wong, J. W. H.; Maleknia, S. D.; Downard, K. M. *J. Am. Soc. Mass Spectrom.,* **2005**, *16*, 225.
[82] Kelleher, N. L. *Anal. Chem.,* **2004**, *76*, 196A.
[83] Iwasaki, H.; Cohen, L. A.; Witkop, B. *J. Am. Chem. Soc.,* **1963**, *85*, 3701.
[84] Iwasaki, H.; Witkop, B. *J. Am. Chem. Soc.,* **1964**, *86*, 4698.
[85] Farber, L.; Cohen, L. A. *Biochemistry,* **1966**, *5*, 1027.
[86] Isoe, S.; Cohen, L. A. *Arch. Biochem. Biophys.,* **1968**, *127*, 522.
[87] MacDonald, S. M.; Roscoe, S. G. *Electrochim. Acta,* **1997**, *42*, 1189.
[88] Permentier, H. P.; Jurva, U.; Barroso, B.; Bruins, A. P. *Rapid Comm. Mass Spectrom.,* **2003**, *17*, 1585.
[89] Permentier, H. P.; Bruins, A. P. *J. Am. Soc. Mass Spectrom.,* **2004**, *15*, 1707.
[90] Burstein, Y.; Patchornik, A. *Biochemistry,* **1972**, *11*, 4641.

[91] Mahoney, W. C.; Hermodson, M. A. *Biochemistry,* **1979**, *18*, 3810.
[92] Steen, H.; Jebanathirajah, J. A.; Rush, J.; Morrice, N.; Kirschner, M. W. *Mol. Cell Proteomics,* **2006**, *5*, 172.
[93] Zhang, T.; Palii, S. P.; Eyler, J. R.; Brajter-Toth, A. *Anal. Chem.,* **2002**, *74*, 1097.
[94] Hayen, H.; Karst, U. *Anal. Chem.,* **2003**, *75*, 4833.
[95] Van Berkel, G. J.; Quirke, J. M. E.; Tigani, R. A.; Dilley, A. S.; Covey, T. R. *Anal. Chem.,* **1998**, *70*, 1544.
[96] Diehl, G.; Liesener, A.; Karst, U. *Analyst,* **2001**, *126*, 288.
[97] Rohner, T. C.; Girault, H. H. *Rapid Comm. Mass Spectrom.,* **2005**, *19*, 1183.
[98] Keller, D. W.; Ponton, L. M.; Porter, M. D. *J. Chromatogr. A,* **2005**, *1089*, 72.
[99] Deng, H.; Van Berkel, G. J.; Takano, H.; Gazda, D.; Porter, M. D. *Anal. Chem.,* **2000**, *72*, 2641.
[100] Pretty, J. R.; Deng, H.; Goeringer, D. E.; Van Berkel, G. J. *Anal. Chem.,* **2000**, *72*, 2066.
[101] Lapizco-Encinas, B. H.; Pinto, N. G. *J. Sep. Sci.,* **2004**, *27*, 667.
[102] Vollmer, S.; Astorga-Wells, J.; Bergman, T.; Jörnvall, H. *Int. J. Mass Spectrom.,* **2007**, *259*, 73.
[103] Nyholm, L. *Analyst,* **2005**, *130*, 599.
[104] Mengeaud, V.; Bagel, O.; Ferrigno, R.; Girault, H. H.; Haider, A. *Lab. Chip,* **2002**, *2*, 39.
[105] Erichsen, T.; Reiter, S.; Ryabova, V.; Bonsen, E. M.; Schuhmann, W.; Märkle, W.; Tittel, C.; Jung, G.; Speiser, B. *Rev. Sci. Instrum.,* **2005**, *76*.

Received: 07 May, 2007 **Revised: 15 August, 2007** **Accepted: 17 August, 2007**